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Butyric acid and hydrogen production by *Clostridium tyrobutyricum* ATCC 25755 and mutants

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Abstract

Clostridium tyrobutyricum produces butyric acid, acetic acid, hydrogen and carbon dioxide as its main fermentation products. In this work, mutants with inactivated *pta* gene, encoding phosphotransacetylase (PTA) and *ack* gene, encoding acetate kinase (AK), were studied for their potential to improve butyric acid production in the fermentation. PTA and AK are two key enzymes in the acetate-producing pathway. PTA and AK activities in the *pta*-deleted mutant (PPTA-Em) were reduced by 44% and 91%, respectively, whereas AK activity in the *ack*-deleted mutant (PAK-Em) decreased by 50%. Meanwhile, the activity of butyrate kinase (BK) in PPTA-Em increased by 44% and hydrogenase activity in PAK-Em increased by 40%. As compared with the wild type, the specific growth rate of the mutants decreased by 32% (from 0.28 to 0.19 h^{-1}) because of the impaired PTA-AK pathway. Meanwhile, butyric acid production by these mutants was improved greatly, with higher butyric acid yield (>0.4 g/g versus 0.34 g/g) and final concentration (43 g/L versus 29 g/L), which also indicated that the mutants had better tolerance to butyric acid inhibition. However, acetate production in the mutants was not significantly reduced even though more butyrate was produced from glucose, suggesting the existence of additional acetate forming pathway in *C. tyrobutyricum*. Also, hydrogen production by PAK-Em mutant increased significantly, with higher hydrogen yield (2.61 mol/mol glucose versus 1.35 mol/mol glucose) and H_2/CO_2 ratio (1.43 versus 1.08). The SDS-PAGE also showed significantly different expression levels of proteins with molecular mass around 32 and 70 kDa. These results suggested that integrational mutagenesis resulted in global metabolic shift and phenotypic changes, which also improved production of butyric acid and hydrogen from glucose in the fermentation.

Keywords: *Clostridium tyrobutyricum*; Butyric acid; Hydrogen; Phosphotransacetylase; Acetate kinase; Hydrogenase

1. Introduction

Clostridium tyrobutyricum is a Gram-positive, rod-shaped, spore-forming, obligate anaerobic bacterium that produces butyric acid, acetic acid, hydrogen and carbon dioxide as its main fermentation products from various carbohydrates including glucose and xylose [1]. Butyric acid is used to synthesize butyryl polymers in the chemical industry and to enhance butter-like note in food flavors in the food industry. Esters of butyrate are used as additives for increasing fruit fragrance and as aromatic compounds for production of perfumes. Butyric acid, as one of the short-chain fatty acids generated by anaerobic fermentation of dietary substrates, is known to have therapeutic effects on colorectal cancer and hemoglobinopathies [2]. The production of butyric acid from renewable resources has become an increasingly attractive alternative to the current petroleum-based chemical synthesis because of public concerns on the environmental pollution caused by the petrochemical industry and consumer's preference to bio-based natural ingredients for foods, cosmetics and pharmaceuticals. Recently, we have demonstrated that butyric acid can be better produced by *C. tyrobutyricum* immobilized in a fibrous-bed bioreactor [3,4]. However, for economical production of butyric acid from biomass, it is desirable to further improve the fermentation process in its final product yield and concentration. It is also desirable to reduce acetate production in the fermentation in order to

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facilitate the separation and purification of the final product, butyric acid.

Hydrogen, with a high-energy content per unit weight (141.86 kJ/g or 61,000 Btu/lb), can be used as a clean fuel and easily converted to electricity by fuel cells. Hydrogen is thus considered as the most promising future fuel if its production cost can be greatly reduced [5]. Hydrogen can be generated in several ways, including catalytic fuel reforming and electrolysis of water. However, the present chemical routes of hydrogen production are energy intensive and expensive as compared with fossil fuels. On the other hand, biological production of hydrogen, either by photosynthesis with algae and photosynthetic bacteria or by fermentation with anaerobic bacteria, can be operated at ambient temperature and pressure [6,7]. Hydrogen production by anaerobic fermentation offers an attractive method to produce energy when low-cost renewable biomass is available as the feedstock [8]. Hydrogen, as an energy byproduct from the butyric acid fermentation, can add value to the fermentation process.

To improve the production of butyric acid and hydrogen by *C. tyrobutyricum*, two metabolically engineered mutants with inactivated acetate kinase gene (*ack*) and phosphotransacetylase gene (*pta*) were created by insertional inactivation of the gene(s) on the chromosome by homologous recombination with integrational plasmids [9]. It was expected that by knocking out *ack* and *pta* genes, the acetate formation pathway would be impaired, resulting in global changes in the metabolic pathway leading to more production of butyric acid and possibly hydrogen. The main objective of this study was thus to evaluate and characterize these *C. tyrobutyricum* mutants for their ability to produce butyric acid and hydrogen from glucose. In this work, kinetics of cell growth, acid production and hydrogen production in fed-batch fermentations by the mutants and wild type of *C. tyrobutyricum* were studied and compared. The activities of phosphotransacetylase (PTA), acetate kinase (AK), phosphotransbutyrylase (PTB), butyrate kinase (BK) and hydrogenase in the mutants were also studied to better understand the effects of *ack* and *pta* inactivation on global changes in acetic acid, butyric acid and hydrogen formation pathways.

2. Materials and methods

2.1. Cultures and medium

C. tyrobutyricum ATCC 25755 was maintained on reinforced clostridial medium (RCM; Difco) plates in an anaerobic chamber (95% N_2, 5% H_2). Working cultures were grown at 37 °C in a previously described synthetic medium (CGM) with glucose as the substrate [10]. Two mutants, PPTA-Em and PAK-Em, were obtained by transforming *C. tyrobutyricum* competent cells with non-replicative plasmids containing erythromycin (Em) resistant gene and partial *pta* or *ack* gene DNA fragment obtained from PCR amplification. The *ack* and *pta* knock-out mutants, created through homologous recombination of the plasmids with the chromosome, were selected and stored on the RCM plates containing 40 μg/mL Em. Details about the construction of the non-replicative plasmids and procedures to obtain the mutants have been given elsewhere [9].

2.2. Fermentation kinetic studies

Fed-batch fermentations of *C. tyrobutyricum* were performed in 5-L stirred-tank fermentors (Marubishi MD-300) containing 2 L of the medium with glucose (∼35 g/L) as the substrate. Anaerobiosis was reached by sparging the medium with nitrogen (10 mL/min) for 30 min. The medium pH was adjusted to ∼6.0 with 6N HCl before inoculation with ∼100 mL of cell suspension prepared in a serum bottle. Experiments were carried out at 37 °C, 150 rpm and pH 6.0 ± 0.1 controlled by adding 5N NaOH solution. The fermentation was operated at a fed-batch mode by manually feeding a concentrated glucose solution whenever the sugar level in the fermentation broth was close to zero. The fermentation was continued until it ceased to produce butyrate due to product inhibition. The production of hydrogen and carbon dioxide in the fermentation was monitored using an on-line respirometer system equipped with H_2 and CO_2 sensors (Micro-oxymax, Columbus Instrument). Liquid samples were taken at regular intervals for the analysis of cell, substrate and product concentrations. Cell density was analyzed by measuring the optical density of cell suspension at 600 nm (OD_{600}) with a spectrophotometer (Sequoia-turner, Model 340). The concentrations of glucose, butyrate and acetate in the liquid samples were analyzed by high performance liquid chromatography (HPLC).

2.3. Preparation of cell extract and enzyme assays

Cells cultivated in 100 mL of CGM at 37 °C were allowed to grow to the exponential phase (OD_{600} = ∼1.5), and then harvested and washed. The cell pellets suspended in 10 mL of 25 mM Tris–HCl buffer (pH 7.4) were sonicated, and the protein extract was collected by centrifugation. All these were done under ambient conditions. The protein extract was then used in AK, BK, PTA and PTB activity assays and SDS-PAGE experiment. For hydrogenase activity assay, cells were suspended in 1 mL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and lysed at 37 °C for 30 min with mutanolysin (100 μg/mL; Sigma). The cell debris was then removed by centrifugation. All procedures for the hydrogenase assay were carried out in the anaerobic chamber. The protein content in the cell extract sample was determined following standard Bradford protocol (Bio-Rad, Hercules, CA).

The activities of PTA and PTB were measured spectrophotometrically at 405 nm by detecting the liberation of CoA from acetyl-CoA and butyryl-CoA, respectively, following the protocol of Andersch et al. [11]. One unit of PTA or PTB activity is defined as the amount of enzyme converting 1 μmol of acetyl-CoA or butyryl-CoA per minute. The activ-

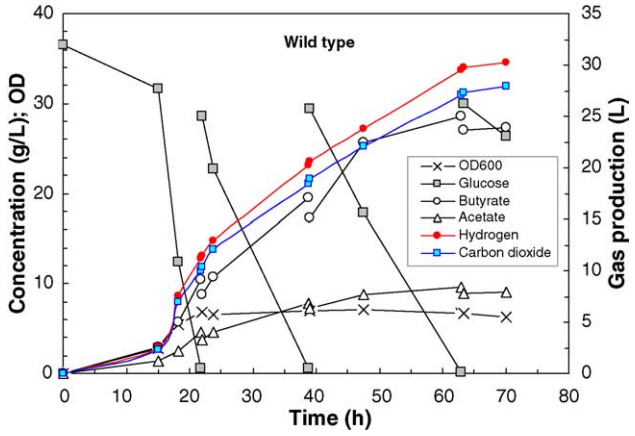

Fig. 1. Kinetics of fed-batch fermentation of glucose by *C. tyrobutyricum* ATCC 25755 wild type at 37 °C, pH 6.0. Legend: OD_{600} (×), glucose concentration (■), butyrate concentration (◯), acetate concentration (Δ), hydrogen volume (●), carbon dioxide volume (▢).

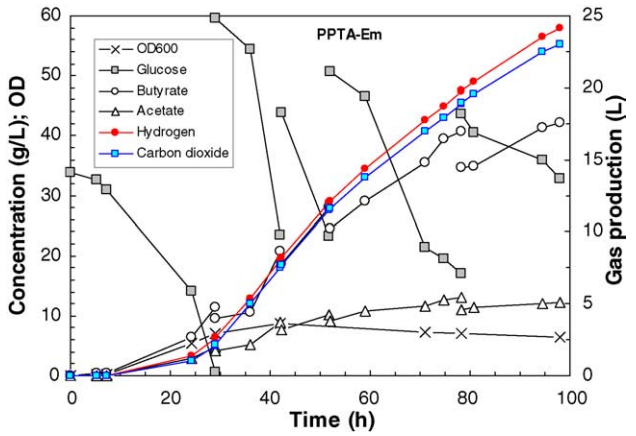

Fig. 2. Kinetics of fed-batch fermentation of glucose by PPTA-Em mutant at 37 °C, pH 6.0. Legend: OD_{600} (×), glucose concentration (■), butyrate concentration (◯), acetate concentration (Δ), hydrogen volume (●), carbon dioxide volume (▢).

ities of AK and BK were assayed using potassium acetate and sodium butyrate as substrate, respectively, by the method of Rose [12]. One unit of AK or BK is defined as the amount of enzyme producing 1 μmol of hydroxamic acid per minute. Hydrogenase activity was detected using the procedure developed by Drake [13]. One unit of hydrogenase activity is defined as 2 μmol of methyl viologen reduced (equivalent to 1 μmol of H_2 oxidized) per minute. Specific enzyme activity was calculated as the units of activity per milligram of protein. The specific enzyme activities in the mutants as compared with the corresponding specific enzyme activities of the wild type were reported as the relative enzyme activity (%) in this work.

2.4. SDS polyacrylamide gel electrophoresis

Protein samples for SDS-PAGE were prepared from the cell extract after sonication and centrifugation. The cell extract (10 mL) was concentrated using four volumes of acetone (40 mL) to precipitate protein at −20 °C overnight, and re-dissolved in 2 mL of 25 mM Tris–HCl buffer (pH 7.4), following the standard protocol (Bio-Rad). Protein samples, 24 μg per well, were loaded into 12.5% SDS-PAGE gel and run at 100 V for 2.5 h with PROTEAN II xi Cell (Bio-Rad) and stained following the instruction of the manufacturer.

3. Results and discussion

3.1. Fermentation kinetics

The kinetics of fed-batch fermentations of glucose with *C. tyrobutyricum* wild type and mutants are shown in Figs. 1–3, respectively. As can be seen in these figures, the wild type grew immediately after inoculation and reached the stationary phase by the end of the first batch, whereas the mutants grew slower and had a lag phase. Production of gas (H_2 and CO_2) and acid products (acetic acid and butyric acid) was low at the beginning but increased afterwards and continued in the stationary phase. It is noted that acetic acid production leveled off much sooner than butyric acid in the fermentation. The fermentations were stopped when glucose was no longer consumed by the cells due to inhibition by butyric acid [3]. However, both mutants showed a better tolerance to butyric acid, as indicated by the much higher final butyrate concentration attained in the fermentations with these mutants than with the wild type. Comparison of the mutants with the wild type in terms of cell growth rate and product yields from glucose is summarized in Table 1 and discussed in the following sections.

3.1.1. Cell growth

The fermentation data showed significant changes in cell growth kinetics of *C. tyrobutyricum* mutants. As shown in Fig. 4, both mutants had a significantly lower specific

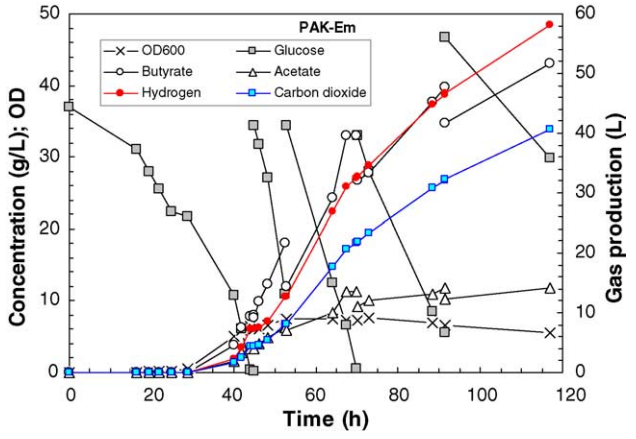

Fig. 3. Kinetics of fed-batch fermentation of glucose by PAK-Em mutant at 37 °C, pH 6.0. Legend: OD_{600} (×), glucose concentration (■), butyrate concentration (◯), acetate concentration (Δ), hydrogen volume (●), carbon dioxide volume (▢).

Table 1
Comparison of fed-batch fermentations of glucose by *C. tyrobutyricum* wild type and mutants at 37 °C, pH 6.0

	Wild type	PPTA-Em	PAK-Em
Cell growth			
Specific growth rate, μ (h^{-1})	0.28 ± 0.03	0.19 ± 0.02	0.19 ± 0.02
Cell biomass yield (g/g)	0.13	0.15	0.11
Acid production			
Final butyric acid concentration (g/L)	28.6	42.1	43.0
Butyric acid yield (g/g)	0.34 ± 0.01	0.40 ± 0.02	0.47 ± 0.03
Final acetic acid concentration (g/L)	9.7	12.2	11.9
Acetic acid yield (g/g)	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
Butyrate/acetate ratio (g/g)	2.96	3.19	3.74
Gas production			
H_2 yield (g/g)	0.015	0.012	0.029
CO_2 yield (g/g)	0.305	0.251	0.446
H_2/CO_2 ratio (mol/mol)	1.08	1.06	1.43

growth rate ($\mu = 0.19 \pm 0.02\,h^{-1}$) as compared to the wild type ($\mu = 0.28 \pm 0.03\,h^{-1}$). It is noted that PAK-Em mutant had a particularly long lag phase of ~10 h. This long lag phase might have been partially attributed to the lower inoculation amount used in the fermentation. The lower specific growth rate for the mutants PPTA-Em and PAK-Em can be attributed to the metabolic burden on cells caused by possibly less energy (ATP) generation in the glucose metabolism due to knock-out of *ack* and *pta*. The cell biomass yield from glucose for the mutants also varied from the wild type. The different biomass yield and specific growth rate of the mutants

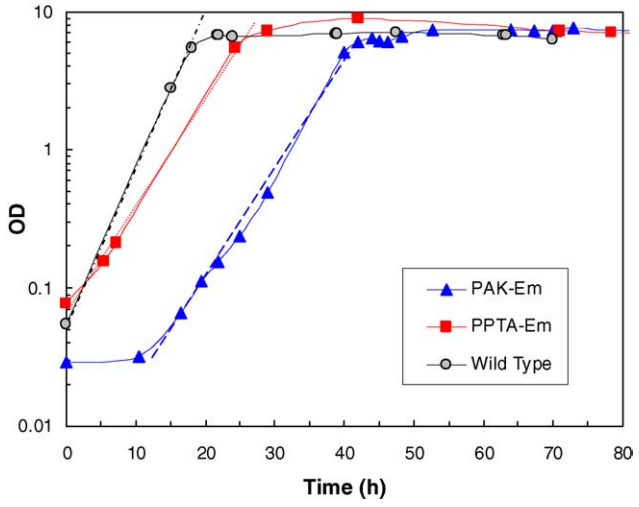

Fig. 4. Cell growth kinetics for the wild type and mutants of *C. tyrobutyricum*. The specific growth rate was estimated from the slope of the semilogarithmic plot. Legend: wild type (●), PPTA-Em (▲), PAK-Em (□).

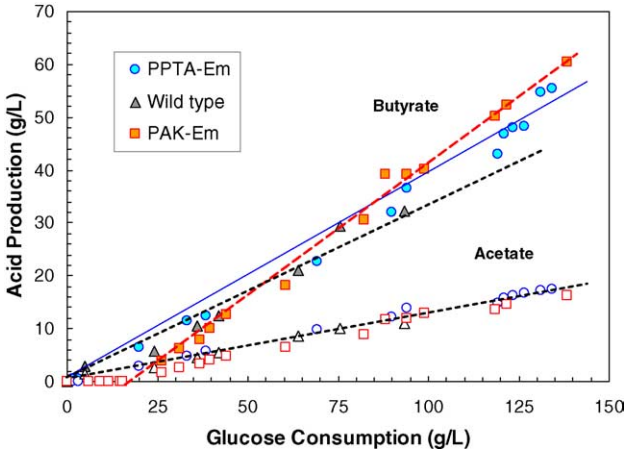

Fig. 5. Comparison of acid production from glucose by the wild type and mutants of *C. tyrobutyricum*. The yields of butyric acid and acetic acid were estimated from the slopes of the linear plots. Legend: wild type (▲), PPTA-Em (●), PAK-Em (□).

indicated that the carbon and energy flux were redistributed in the metabolic pathways of the mutants, which also resulted in significant changes in production of various fermentation products.

3.1.2. Acid production

Butyric acid production in the fermentation greatly improved in both final product concentration and yield in the mutants. As can be seen in Table 1, the final concentration of butyric acid produced in the fed-batch fermentations increased by ~50%, from 28.6 g/L by the wild type to 42.1 g/L by PPTA-Em and 43.0 g/L by PAK-Em. This result suggested that the butyric acid tolerance of the *pta*-deleted mutant (PPTA-Em) and *ack*-deleted mutant (PAK-Em) was greatly enhanced to allow them to produce more butyric acid. Furthermore, the butyric acid yield from glucose also increased significantly, from 0.34 g/g by the wild type to 0.40 g/g by PPTA-Em and 0.47 g/g by PAK-Em. It is noted that the butyrate yield for PAK-Em would have been higher (~0.51 g/g) if the glucose consumption during the lag phase was neglected (see Fig. 5). However, acetic acid production in the mutants was similar to that in the wild type. As can be seen in Fig. 5, acetic acid yield from glucose was almost identical (~0.12 g/g) for all three strains studied. Nevertheless, the butyrate/acetate ratio (g/g) increased from 2.96 for the wild type to 3.19 for PPTA-Em and 3.74 for PAK-Em, a clear indication that the metabolic pathway in the mutants had been shifted to favor butyric acid production over acetic acid production. This metabolic shift can be attributed to the mutations resulted from the knock out of *ack* and *pta* genes in the mutants, which will be further discussed later in this paper.

3.1.3. Gas production

As can be seen in Figs. 1–3, hydrogen and carbon dioxide were produced simultaneously throughout the fermentation.

Table 2
Enzyme activities in mutants of *C. tyrobutyricum* as compared to the wild type

	PTA	AK	PTB	BK	Hydrogenase
PPTA-Em (%)	56 ± 0.7	9 ± 0.7	99 ± 5.7	144 ± 1.6	99 ± 2.5
PAK-Em (%)	142 ± 1.4	50 ± 0.9	97 ± 0.8	97 ± 0.1	140 ± 3.2

Gas production initially was low, but increased rapidly in the exponential and stationary phases. The hydrogen yield was 0.015 g/g or 1.35 mol/mol glucose for the wild type, 0.012 g/g or 1.08 mol/mol for PPTA-Em and 0.029 g/g or 2.61 mol/mol for PAK-Em (Table 1). It is noted that the hydrogen production was slightly lowered in PPTA-Em but increased dramatically in PAK-Em. The mole ratio between hydrogen and carbon dioxide was 1.08 for the wild type and 1.06 for PPTA-Em, but increased to 1.43 for PAK-Em. Apparently, more electrons were released from the oxidation reaction of FdH_2 to Fd and transferred to H^+ to produce more hydrogen in PAK-Em. It is known that hydrogen production in anaerobic fermentation could be greatly affected by various operation factors such as nutrient level, stirring speed, pH and temperature [8]. In this work, however, all fermentations were carried out under similar conditions. The higher hydrogen production in PAK-Em was in fact resulted from the higher hydrogenase activity in the mutant (see Table 2), which was unexpected in the gene manipulation experiment.

3.2. Protein expression and enzyme activities

To better understand the fermentation kinetics changes resulted from mutations generated from inactivating *ack* and *pta* genes in the central metabolic pathway of *C. tyrobutyricum*, protein expressions in the mutants and wild type were studied and compared. Fig. 6 shows the results from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two notable differences between the wild type and the mutants can be seen in the SDS-PAGE gel map. First, the protein with the molecular mass of ~32 kDa was highly expressed in the wild type but was low in both PPTA-Em and PAK-Em mutants. Second, the protein with ~70 kDa molecular mass was much higher for the PAK-Em mutant than the wild type. This 70 kDa protein has the molecular mass similar to that of the hydrogenase in a similar species, *C. acetobutylicum*, which was reported to be ~67 kDa [14]. Thus, this protein is likely to be the hydrogenase in *C. tyrobutyricum*. This finding is consistent with the much increased hydrogenase activity and hydrogen production by the mutant PAK-Em. Further experiments with on-gel hydrogenase activity assay [15,16] could provide a direct evidence of the identity of this 70 kDa protein. The missing 32 kDa proteins in the mutants likely belong to phosphotransacetylase (PTA) and acetate kinase (AK), although it is not possible to identify PTA and AK on the SDS-PAGE gel map due to limited proteomics information for *C. tyrobutyricum* and similar species. AK and PTA from several microorganisms have been characterized, but the results showed large variations in their molecular mass [17]. Enzyme activity assays were thus carried out to further study hydrogenase and various key enzymes in the acid forming pathways (see Fig. 7).

The specific enzyme activities for PTA, AK, PTB, BK and hydrogenase in PPTA-Em and PAK-Em mutants were assayed and their relative activities as compared with those of the wild type are summarized in Table 2. For PPTA-Em, the PTA activity was reduced by 44% and AK decreased by 91%, whereas BK activity increased by 44% and PTB and hydrogenase unchanged. Compared to the wild type, the PAK-Em mutant had a lower AK activity (50%) but unexpectedly higher activities in PTA (142%) and hydrogenase (140%), and similar activities in PTB and BK (97%). The activities of both PTA and AK in the *pta*-deleted mutant (PPTA-Em) were decreased dramatically, but only AK activity in the *ack*-deleted mutant (PAK-Em) was reduced, indicating that the *pta* gene lies upstream from *ack* gene in the same operon. However, both mutants with the intended *ack* or *pta* gene knock-out did not completely eliminate acetate production as there were significant residual activities of PTA and AK in these mutants. It is noted however, that there may be some other enzymes, besides PTA and AK, present in *C. tyrobutyricum* that also can use the same (acetyl-CoA)

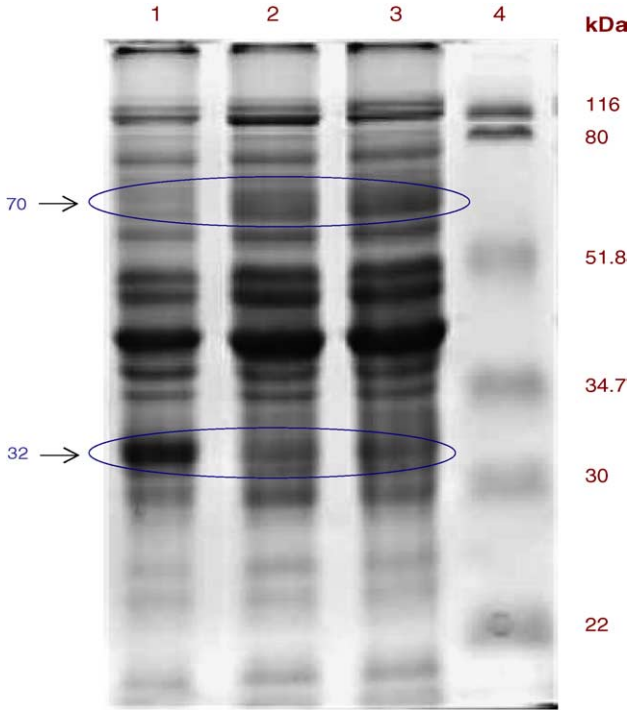

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of cellular proteins from *C. tyrobutyricum* (lane 1: wild type; lane 2: PPTA-Em mutant; lane 3: PAK-Em mutant; lane 4: molecular marker.).

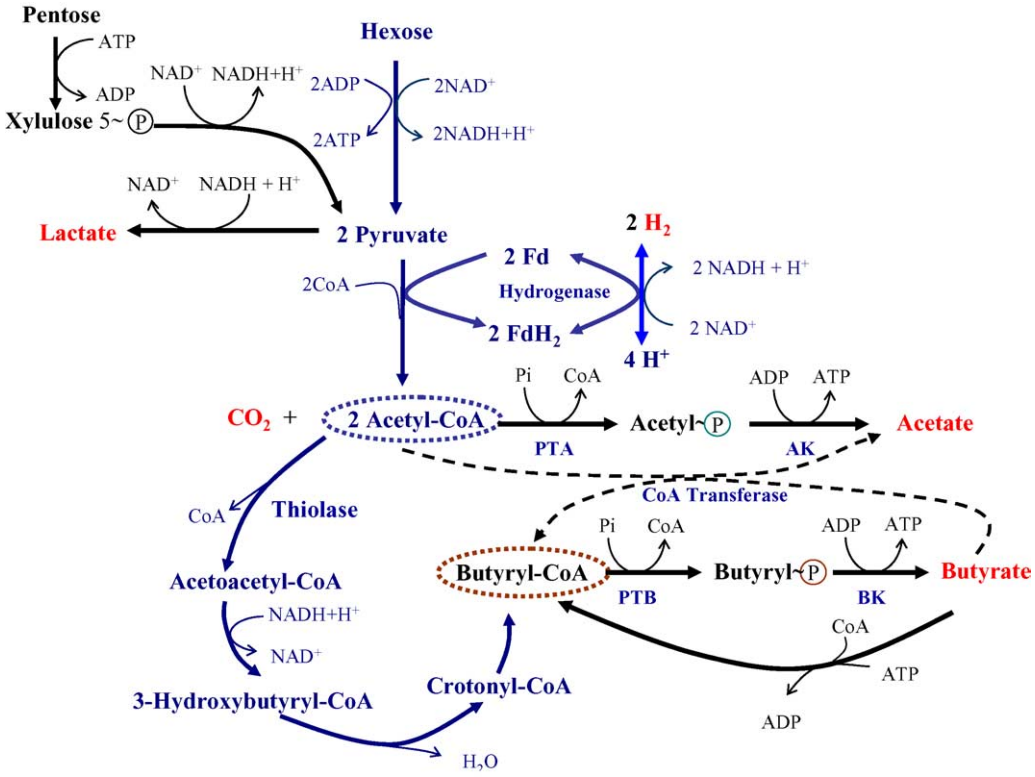

Fig. 7. The metabolic pathway in *Clostridium tyrobutyricum* ATCC 25755. The enzymes studied in this work include: hydrogenase, phosphotransacetylase (PTA), acetate kinase (AK), phosphotransbutyrylase (PTB) and butyrate kinase (BK). The dashed lines indicate a possible reaction catalyzed by CoA transferase to produce acetate in the absence of PTA and AK.

and perhaps other substrates to produce acetic acid. The effects of (partial) inactivation of *ack* and *pta* in the mutants on the key enzymes in the central metabolic pathway and fermentation kinetics are further discussed in the following section.

3.3. Effects of integrational mutagenesis

Fig. 7 shows the central metabolic pathway in *C. tyrobutyricum*. In general, glucose (hexose) is catabolized via Embden-Meyerhof-Parnas (EMP) pathway and xylose (pentose) is catabolized by Hexose Monophosphae (HMP) pathway to pyruvate, which is then oxidized to acetyl-CoA and carbon dioxide with concomitant reduction of ferredoxin (Fd) to FdH_2. FdH_2 is then oxidized to Fd to produce hydrogen, catalyzed by hydrogenase, with excess electron released to convert NAD^+ to NADH [18]. Acetyl-CoA is the key metabolic intermediate at the node dividing the acetate-formation branch from butyrate-formation branch. Phosphotransacetylase (PTA) and acetate kinase (AK) are two enzymes that convert acetyl-CoA to acetic acid, whereas phosphotransbutyrylase (PTB) and butyrate kinase (BK) catalyze the production of butyric acid from butyryl-CoA. In this work, two mutants with (partially) inactivated *ack* and *pta* were created by insertional inactivation of the gene(s) on the chromosome by homologous recombination with integrational plasmids containing partial DNA sequences of *ack* and *pta* genes, respectively. Both fermentation kinetics and enzyme activity assay results indicated that inactivation of *ack* and *pta* genes in the acetic acid formation pathway of *C. tyrobutyricum* led to global changes in additional key enzymes in the whole metabolic pathway network. As discussed before, the activities of acetic acid-forming enzymes, AK and PTA, corresponding to the targeted genes *ack* and *pta*, in the PAK-EM and PPTA-Em mutants decreased greatly, while BK in PPTA-Em and hydrogenase and PTA in PAK-Em increased significantly.

Since AK and PTA activities were significantly reduced in the mutants, more pyruvate must have been catabolized through the butyrate-producing pathway, leading to higher butyrate yields from glucose. For the same reason, the mutants suffered from a slower growth rate due to less ATP was produced from the acetate-producing (PTA-AK) pathway, which can generate more ATP per mole of glucose metabolized than the butyrate-producing (PTB-BK) pathway can. Interestingly, to compensate for the lost energy efficiency due to reduced flux through the PTA-AK pathway, PPTA-Em mutant increased its BK activity and PAK-Em mutant increased its hydrogenase activity. The increased hydrogenase activity not only resulted in more hydrogen production, but also might have increased the amount of NADH and energy production. The increase of PTA activity in PAK-Em mutant was probably due to the positive control on protein expression by the accumulation of acetyl-CoA

resulted from the (partial) inactivation of the PTA-AK pathway.

The final butyric acid concentration produced by *pta*-deleted mutant (PPTA-Em) and *ack*-deleted mutant (PAK-Em) increased greatly as compared with that of the wild type. The increased butyric acid tolerance of the mutants also may be attributed to the reduced flux through the PTA-AK pathway. Our previous study has shown that the acetic acid-forming enzymes (PTA and AK) are more sensitive to butyric acid inhibition than butyric acid-forming enzymes (PTB and BK) [3]. Since the mutants were no longer dependent on the PTA-AK pathway for energy production and survival, they became less sensitive to butyric acid inhibition.

However, acetic acid production in PPTA-Em and PAK-Em mutants was not significantly affected in the fermentations even though the mutants had a much lower AK activity and the PTA-AK pathway should have been impaired. In fact, the final acetic acid concentration produced by the mutants in the fermentations was higher than that by the wild type. Apparently, besides PTA and AK, there are other enzymes in *C. tyrobutyricum* that can also produce acetate from acetyl-CoA and perhaps other substrates as well. For example, CoA transferase can catalyze the formation of acetate from acetyl-CoA. This enzyme has been found in some clostridia bacteria [18] and could also be present in *C. tyrobutyricum*. Possible presence of PTA and AK isozymes also could not be ruled out. Two acetate kinase isozymes from spirochete MA-2 cell extracts [19] and a butyrate kinase isozyme (BKII) in *C. acetobutylicum* ATCC 824 [20] have been reported. Thus, it can be concluded that the acetic acid-producing pathway in *C. tyrobutyricum* is more complex than what we currently know and acetic acid production may be critical to cell's survival.

4. Conclusions

Phosphotransacetylase gene (*pta*) and acetate kinase gene (*ack*) encode two important enzymes involving in the metabolic pathway to form acetic acid from acetyl-CoA, which plays an important role for the metabolic flux distribution of carbon and energy. Two mutants of *C. tyrobutyricum* obtained from integrational mutagenesis to selectively inactivate *ack* and *pta* genes were studied for their fermentation kinetics. As compared with the wild type, butyric acid production by these mutants was improved with higher final product concentration and yield. It is clear that more carbon and energy fluxes can flow into the metabolic pathway leading to the production of butyric acid in the mutants. Also, these mutants have better tolerance to butyric acid inhibition. The *ack*-deleted mutant also has improved hydrogen production. This study demonstrates that butyric acid and hydrogen production from glucose can be significantly improved by using metabolically engineered mutants of *C. tyrobutyricum*. Increasing the final butyric acid concentration, yield and volumetric productivity in the fermentation should reduce the production cost for bio-based butyric acid and allow the bioproduction method to compete more favorably in the marketplace.

Acknowledgements

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